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Evidence That a Hemoglobin Adduct of 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone Is a 4-(3-Pyridyl)-4-oxobutyl Carboxylic Acid Ester

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Hemoglobin adducts of the carcinogenic tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) release 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) upon mild base or acid hydrolysis. HPB has been detected in hydrolysates of human hemoglobin and has been proposed as a dosimeter of exposure to and metabolic activation of NNK in people exposed to tobacco products. In this study, labeling experiments were carried out with $Na^{18}OH$ which provide strong evidence that the globin adduct which releases HPB upon base hydrolysis is a carboxylic acid ester. Globin was isolated from rats treated with NNK. This globin was reacted with $NaCNBH_3$, followed by hydrolysis at room temperature with 0.2 N NaOH. Analysis of the products demonstrated the presence of 4-hydroxy-1-(3-pyridyl)-1-butanol (7), but not HPB. These results demonstrate that the adduct in globin has a free carbonyl group and is not a Schiff base. This sequence of reactions was then carried out with $Na^{18}OH$, under conditions which would have resulted in incorporation of ^{18}O into 7 if nucleophilic displacement at carbon 4 of the adduct had occurred. Analysis of the products by GC–MS showed no detectable incorporation of ^{18}O into 7. These results demonstrate that the globin adduct which releases HPB upon base hydrolysis is a 4-(3-pyridyl)-4-oxobutyl carboxylic ester. Consistent with this conclusion, a model ester, α-methyl β-[4-(3-pyridyl)-4-oxobutyl] *N*-(carbobenzyloxy)-L-aspartate (13), hydrolyzed in base and acid in a manner similar to that observed with globin from NNK-treated rats.

Introduction

When rats or mice are treated with the carcinogenic tobacco-specific nitrosamine NNK[1] (1), hemoglobin adducts are formed (*1–3*). Mild base hydrolysis of this hemoglobin releases 20–40% of the bound material as HPB (5) (Figure 1). Release of HPB from human hemoglobin has been suggested as a dosimeter of exposure to, and metabolic activation of, NNK, as well as a related nitrosamine, *N'*-nitrosonornicotine. Levels of HPB released from hemoglobin have been quantified in humans by GC–MS (*4*).

Figure 1 illustrates the postulated mechanism of NNK–hemoglobin adduct formation. Enzymatic hydroxylation of the methyl carbon yields α-(hydroxymethyl)-NNK (3), which is unstable and spontaneously decomposes to formaldehyde and 4-(3-pyridyl)-4-oxobutanediazohydroxide (4). This intermediate reacts with a nucleophilic site in hem-

[1] Abbreviations: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone; GC–MS, gas chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; NNK-*N*-oxide, 4-(methylnitrosamino)-1-(*N*-oxy-3-pyridyl)-1-butanone; NNAL-*N*-oxide, 4-(methylnitrosamino)-1-(*N*-oxy-3-pyridyl)-1-butanol; BSTFA, bis(trimethylsilyl)trifluoroacetamide; TMS, trimethylsilyl; TLC, thin-layer chromatography; CI-MS, chemical ionization mass spectrum; EI-MS, electron impact mass spectrum.

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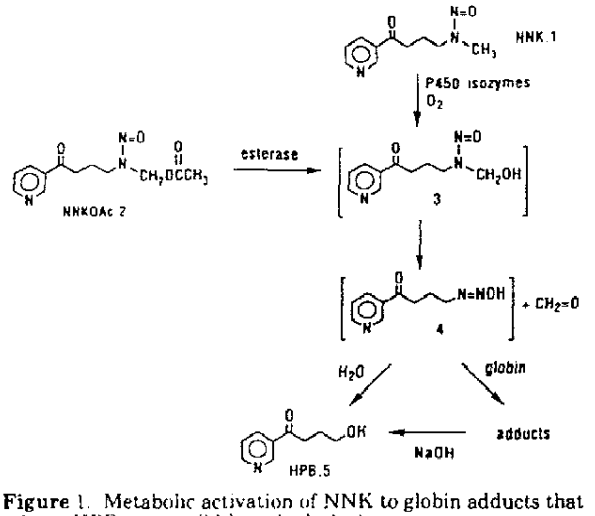

Figure 1. Metabolic activation of NNK to globin adducts that release HPB upon mild base hydrolysis.

oglobin to produce an adduct which releases HPB upon hydrolysis of the hemoglobin in dilute NaOH or HCl at room temperature. Previous studies have provided support for this mechanism. These studies have shown that the adduct is formed by covalent binding to globin (*3*). α-Acetoxy-NNK (*2*), a stable precursor to α-hydroxy-NNK, formed the same adduct upon injection into mice (*3*). In contrast, HPB itself did not react with hemoglobin in vitro and did not produce an HPB-releasing hemoglobin adduct when injected into mice (*3*).

An earlier study showed that cysteine is not a site of pyridyloxobutyl adduct formation in the hemoglobin of rats treated with NNK (*5*). In this paper, we describe experiments with $Na^{18}OH$ designed to test the hypothesis that the HPB-releasing adduct is an ester. Hydrolysis of such an adduct with NaOH would be expected to occur by the $B_{AC}2$ mechanism, which would not result in incorporation of ^{18}O into the released alcohol. Since the HPB-releasing adduct could contain a carbonyl group, which would exchange with $Na^{18}OH$ under these conditions, it was necessary to first treat the adducted hemoglobin with $NaCNBH_3$.

Experimental Section

Apparatus. HPLC was performed with a Waters Associates system (Millipore; Waters Division, Milford, MA), equipped with a Model 440 UV/visible detector operated at 254 nm or a Flo-one/Beta radioactive flow detector (Radiomatic Instruments, Tampa, FL). NMR spectra were obtained with a Bruker Model AM 360 WB spectrometer. MS and GC–MS analyses were carried out with a Hewlett-Packard Model 5988A instrument.

Chemicals. NNK (*1*), HPB (*5*), NNK-*N*-oxide, NNAL-*N*-oxide, and 4-hydroxy-1-(3-pyridyl)-1-butanol (*7*) were synthesized (*6–9*). [5-^{3}H]NNK, which has tritium at the 5-position of the pyridine ring, was obtained from Chemsyn Science Laboratories, Lenexa, KS. $H_2{}^{18}O$, 90% isotopic purity, was purchased from Alfa Products (Ward Hill, MA). $Na^{18}OH$ was prepared by adding Na metal to $H_2{}^{18}O$. *N*-(Carbobenzyloxy)-L-aspartic acid α-methyl ester was purchased from Sigma Chemical Co., St. Louis, MO. BSTFA was purchased from Pierce Chemical Co. (Rockford, IL). All other chemicals used in the synthetic procedures and solvolysis experiments were obtained from Aldrich Chemical Co., Milwaukee, WI.

(A) 4-Acetoxy-1-(3-pyridyl)-1-butanol (8). HPB (2.0 mg, 0.012 mmol) was dissolved in 1 mL of CH_2Cl_2, and the solution was cooled to 0 °C. Triethylamine (6.7 μL, 0.048 mmol) and acetic anhydride (2.6 μL, 0.024 mmol) were added. The solution was stirred at 0 °C, and the reaction was followed by TLC. It was complete after 1 h. The solution was concentrated to dryness, and the residue was redissolved in CH_2Cl_2 and washed with H_2O. The organic layer was dried ($MgSO_4$), and the solvent was evaporated. The residue was dissolved in 1 mL of MeOH, and $NaBH_4$ (2.2 mg, 0.06 mmol) was added. The mixture was stirred at room temperature for 15 min. The MeOH was evaporated, the residue was redissolved in H_2O, the pH was adjusted to 7.0, and the product was extracted with CH_2Cl_2. Drying and evaporating gave *8*: NMR ($CDCl_3$) δ 8.6 (bs, 2, pyr-2 and -6H), 7.77 (m, 1, pyr-4H), 7.35 (m, 1, pyr-5H), 4.83 (m, 1, CHOH), 4.11 (m, 2, CH_2OAc), 2.45 (bs, OH), 2.05 (s, 3, $OCOCH_3$), 1.8 (m, 4, CH_2CH_2); CI-MS, methane (*m/z*, rel intensity) 210 (M + 1, 100), 192 (14), 169 (6), 150 (18), 132 (28). The purity of *8* was greater than 98% by HPLC analysis.

(B) 2-(3-Pyridyl)ethyl *p*-Toluenesulfonate (9). Ethyl 3-pyridylacetate (25 g, 0.15 mol) in 340 mL of Et_2O was stirred at room temperature, and 5.75 g of LAH in 190 mL of Et_2O was added at a rate which maintained gentle refluxing. After the addition was complete, stirring was continued for 45 min. Then 46 mL of saturated aqueous sodium potassium tartrate was added. After reaction was complete, the liquid was decanted and the residue washed thoroughly with CH_2Cl_2. The combined organic extracts were concentrated, and the residue was distilled to give 15.9 g of 2-(3-pyridyl)ethanol as an oil: NMR ($CDCl_3$) δ 8.5 (bs, 2, pyr-2,6H), 7.70 (d, 1, pyr-4H), 7.3 (m, 1, pyr-5H), 3.92 (t, 2, CH_2OH), 2.95 (t, 2, CH_2-pyr).

2-(3-Pyridyl)ethanol (20 mg, 0.163 mmol) was dissolved in 2 mL of CH_2Cl_2 and cooled to 0 °C. Pyridine (53 μL, 0.65 mmol) and *p*-toluenesulfonyl chloride (62.1 mg, 0.326 mmol) were added. The solution was stirred at 0 °C overnight. The CH_2Cl_2 and pyridine were removed by evaporation, and the residue was purified by silica gel chromatography with elution by ethyl acetate, to give the tosylate *9* in 90% yield: NMR ($CDCl_3$) δ 8.48 (dd, 1, pyr-6H), 8.37 (d, 1, pyr-2H), 7.68 (d, 2, Ar-2,6H), 7.47 (ddd, 1, pyr-4H), 7.28 (d, 2, Ar-3,5H), 7.19 (dd, 1, pyr-5H), 4.21 (t, 2, CH_2OTs), 2.95 (t, 2, CH_2-pyr), 2.43 (s, 3, CH_3).

(C) α-Methyl β-[4-(3-Pyridyl)-4-oxobutyl] *N*-(Carbobenzyloxy)-L-aspartate (13). *N*-(Carbobenzyloxy)-L-aspartic acid α-methyl ester (45 mg, 0.16 mmol) was treated with 250 μL of oxalyl chloride. The solution was stirred at room temperature for 3.5 h. The oxalyl chloride was removed under vacuum, and the residue was dissolved in 1 mL of CH_2Cl_2. Triethylamine (50 μL, 0.36 mmol) was mixed with this solution, and it was added to a solution of HPB (6 mg, 0.036 mmol) in 1 mL of CH_2Cl_2. The solution was stirred at room temperature overnight and then concentrated to dryness. The residue was redissolved in CH_2Cl_2 and washed with H_2O. The organic layer was dried ($MgSO_4$) and evaporated, yielding a residue which was purified by silica gel chromatography with elution by ethyl acetate. The product **13** was obtained in 80% yield: NMR ($CDCl_3$) δ 9.20 (bs, 1, pyr-2H), 8.80 (bs, 1, pyr-6H), 8.30 (d, 1, pyr-4H), 7.48 (t, 1, pyr-5H), 7.36 (bs, 5, ArH), 5.75 (m, 1, NH), 5.11 (s, 2, CH_2), 4.65 (m, 1, α-CH), 4.20 (t, 2, CH_2O), 3.75 (s, 3, CH_3O), 3.07 (t, 2, CH_2CO), 3.01 (dd, 1, β-CH), 2.85 (dd, 1, β-CH), 2.09 (p, 2, $CH_2CH_2CH_2$); CI-MS, methane (*m/z*, rel intensity) 429 (M + 1, 100), 321 (26), 148 (36), 91 (86).

Solvolyses of Hydroxy Acetate 8 and Tosylate 9 with $Na^{18}OH$. Hydroxy acetate *8* (0.5 mg, 0.0024 mmol) was treated with 0.1 mL of 0.5 N $Na^{18}OH$ for 1 h at room temperature. TLC analysis (EtOAc) showed that the reaction was complete. The solution was neutralized with 1 N HCl and extracted with CH_2Cl_2. The extract was dried and evaporated. The residue consisted of 4-hydroxy-1-(3-pyridyl)-1-butanol (*7*), which was analyzed for ^{18}O incorporation by CI-MS.

Tosylate *9* (1 mg, 0.0036 mmol) was treated with 0.1 mL of 0.5 N $Na^{18}OH$ solution. Acetonitrile (25 μL) was added to dissolve the tosylate. The solution was stirred overnight at room temperature. The pH was adjusted to 7.0, and the reaction mixture was analyzed by HPLC and TLC. The main product, detected by TLC with elution by ethyl acetate, was 3-vinylpyridine (95%). 2-(3-Pyridyl)ethanol (*10*) was formed in 5% yield and was purified by HPLC on a 4.6 mm by 12.5 cm Partisil 5 ODS 3 column (Whatman, Clifton, NJ) eluted with a linear 50-min gradient from 20 mM sodium phosphate buffer (pH 7.0) to methanol at a flow rate of 1 mL/min. The retention time of **10** was 13.7 min. It was collected and further purified using the following gradient: 100%

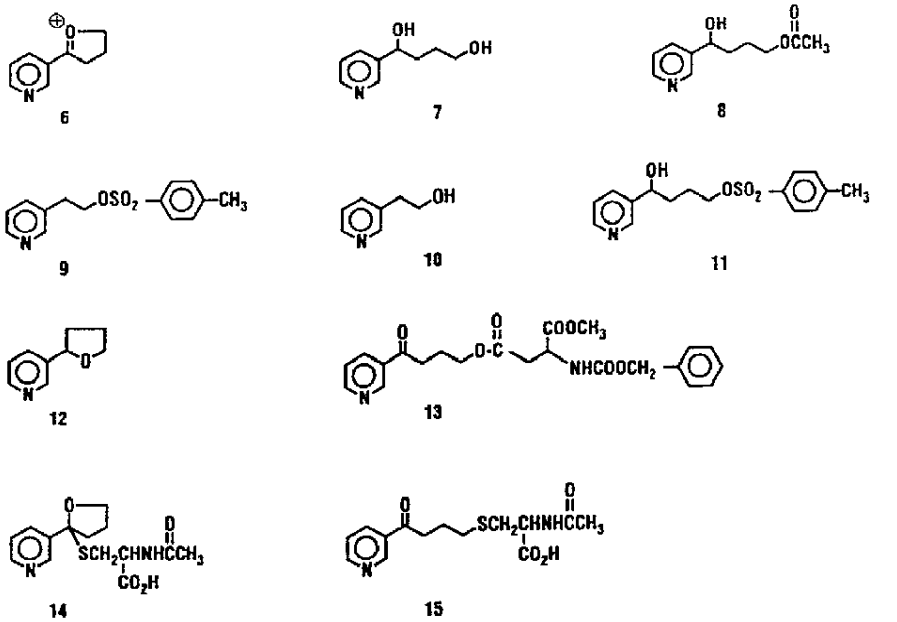

Figure 2. Structures of the compounds discussed in the text.

H_2O for 10 min, and then a linear program to 50% MeOH in H_2O in 10 min at 1 mL/min. The retention time of **10** was 19.9 min. The collected material was analyzed by EI-MS: *m/z* (rel intensity) 125 (M^+, 36), 123 (6), 93 (100), 92 (49). The EI-MS of **10** had *m/z* 123 (M^+, 38), 93 (100), 92 (54).

Treatment of Globin with $NaCNBH_3$ Followed by NaOH. Two male F-344 rats weighing 250–300 g and purchased from Charles River Breeding Laboratories, Kingston, NY, were each given a daily ip injection of [5-^{3}H]NNK (1.67 mCi, 1.72 μmol) in 0.6 mL of saline, for 3 days. Twenty-four hours after the final injection, blood was obtained by cardiac puncture under ether anesthesia. Globin (125 mg) from these rats was isolated as described (*1*) and dissolved in 12.5 mL of 0.1 M potassium phosphate buffer (pH 7.0) containing 16.7 M urea. EDTA (37.5 mg in 0.75 mL) was added, followed by slow addition of 250 mg of $NaCNBH_3$ (*10*, *11*). The solution was stirred at room temperature for 2–3 h. Then 0.5 mL of acetic acid was added, and the solution was dialyzed overnight with three changes of H_2O and freeze-dried. Twenty milligrams of this globin was dissolved in 0.5 mL of 0.2 N NaOH, and the mixture was sonicated for 1 h. The resulting mixture was neutralized and centrifuged, and the supernatant was injected on HPLC using a 4.6 mm × 25 cm Partisphere 5 C-18 reverse-phase column (Whatman). The gradient was 86% A for 40 min and then linear to 100% B in 23 min, at 1 mL/min. Solvent A was 0.025 M sodium acetate buffer (pH 5.5), and solvent B was MeOH.

Treatment of Globin with $NaCNBH_3$ Followed by $Na^{18}OH$. Two male F344 rats weighing 270 g were each given a single ip injection of [5-^{3}H]NNK [(0.2 mCi/100 mg)/kg] in saline and killed 24 h later. Globin (100 mg) from these rats was dissolved in 10 mL of 0.1 M potassium phosphate buffer (pH 7.0) containing 13 M urea. EDTA (30 mg in 0.6 mL of H_2O) was added, followed by addition of $NaCNBH_3$ (300 mg) over a 1-h period. The solution was stirred at room temperature for 2–3 h. The pH remained at 7. Acetic acid (0.5 mL) was added to maintain the pH at 6–7 and to keep the globin in solution. The solution was then dialyzed and freeze-dried as described above. Fifty milligrams of this globin was dissolved in 0.4 mL of 0.5 N $Na^{18}OH$. The solution was sonicated for 2 h and then neutralized with a few microliters of concentrated HCl. Globin precipitates in this step. H_2O (0.5 mL) was added, and the mixture was vortexed and centrifuged.

The supernatant was analyzed by HPLC using the Partisphere 5 C-18 column programmed as follows: 100% A to 65% A in 70 min, and then to 100% B in 10 min. Solvent A was 20 mM sodium phosphate buffer (pH 7.0), and solvent B was MeOH. The retention time of diol **7** was 30.3 min. This retention time was marked for collection from HPLC by adding a few micrograms of NNAL-*N*-oxide (retention time 25.6 min) and NNK-*N*-oxide (retention time 33.8 min) to the sample. The collected band was further purified and desalted by a second HPLC step, using 100% H_2O for 10 min, and then a linear gradient to 50% MeOH in H_2O in 10 min. The retention time of **7** in this system was 23.3 min.

The collected material was concentrated to dryness, and the residue was redissolved in MeOH and transferred to a 250-μL conical glass vial for silylation. The MeOH was evaporated, and 10 μL of BSTFA was added. The solution was kept at room temperature for 2 h and then analyzed by GC–MS using a 12 m × 0.2 mm HP-1 (methyl silicone) column (film thickness 0.33 μm; Hewlett-Packard, Palo Alto, CA) with splitless injection. The injection and detector temperatures were 270 and 275 °C, respectively. The oven was programmed from 35 to 275 °C, at 5 °C/min. The retention time of the bis-TMS ether of **7** was 23.8 min.

Solvolysis of Aspartate Ester 13. Aspartate ester **13** (1 mg) was dissolved in 1 mL of either 0.15 N NaOH or 0.2 N HCl, and the solutions were sonicated for 1 h at room temperature. Following neutralization, they were analyzed by HPLC on the Partisphere 5 C-18 column with a linear 100-min gradient from 0.025 M pH 5.5 sodium acetate buffer to methanol at 1 mL/min. The retention times of **13** and HPB were 65 and 30 min, respectively.

Results

Our initial goal was to determine whether or not the HPB-releasing adduct had a free carbonyl group. The cyclic intermediate **6** (Figure 2) is formed during the solvolysis of NNK-OAc and could be involved in adduct formation, producing an adduct without a carbonyl group (*12*). This information was necessary before ^{18}O labeling experiments could be carried out, since they would be confounded by exchange if a free carbonyl group were present. Globin was isolated from rats treated with [5-^{3}H]-NNK. The globin was allowed to react with $NaCNBH_3$ at pH 6–7, conditions that do not release HPB. The $NaCNBH_3$-treated globin was dialyzed and then hydrolyzed with mild base. The hydrolysate was analyzed by HPLC. As illustrated in Figure 3, about 20% of the radioactivity coeluted with 4-hydroxy-1-(3-pyridyl)-1-butanol (**7**). This was also identified by GC–MS (see below). The

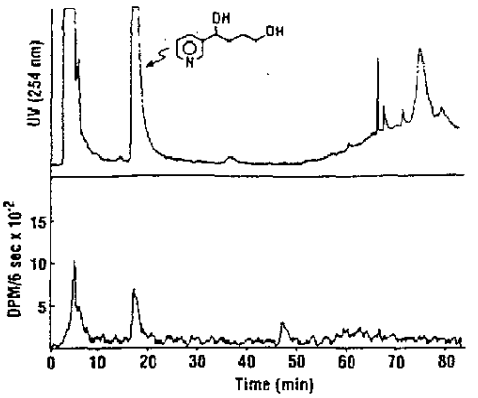

Figure 3. HPLC chromatogram of a 0.2 N NaOH hydrolysate of $NaCNBH_3$-treated globin that had been isolated from a rat injected with [5-^{3}H]NNK. Under these conditions, HPB elutes at 29 min.

identity of the other peaks was not further investigated, but they were not detected in base hydrolysates of globin that had not been treated with $NaCNBH_3$. HPB, which elutes at 29 min under these conditions, was not detected in this sample. When this sample was base-treated without prior reaction with $NaCNBH_3$, 25% of the radioactivity was released as HPB, which is consistent with previous results (*1*, *2*, *5*). These data demonstrate that a free carbonyl group is present in the HPB-releasing adduct, which therefore must be a 4-(3-pyridyl)-4-oxobutyl adduct rather than a cyclic one. The results also showed that the HPB-releasing adduct is not a Schiff base, because this would not release 7 following $NaCNBH_3$ treatment.

The next series of experiments involved hydrolysis with $Na^{18}OH$. These experiments were carried out on globin that had been treated with $NaCNBH_3$, in order to avoid exchange of the carbonyl oxygen. If the adduct were a carboxylic ester, we would expect hydrolysis by the $B_{AC}2$ mechanism and no incorporation of ^{18}O into the diol 7. Hydrolysis of an ether derived from serine, threonine, or tyrosine would not occur under the mild conditions which we are using. Any other adduct between carbon 4 of the diazohydroxide and, for example, histidine, cysteine, arginine, or lysine of globin would hydrolyze with incorporation of ^{18}O into 7.

Conditions for this experiment were developed using two model compounds, the hydroxy ester 8 and the tosylate 9. Compound 8 was a model for a carboxylic ester adduct. As expected, hydrolysis of this compound with $Na^{18}OH$ yielded 7, with an M + 1 peak of m/z 168 (rel intensity 100) in its CI-MS; no peak at m/z 170 was detected. Thus, there was no incorporation of label into the product, diol 7. Compound 9 was a model for adducts which would have undergone a nucleophilic substitution reaction resulting in incorporation of ^{18}O. Hydrolysis with $Na^{18}OH$ produced mainly 3-vinylpyridine (95%). The minor product, 10, contained >85% ^{18}O. We also attempted to prepare hydroxy tosylate 11 as a model, but it was unstable and its solvolysis produced exclusively the furan 12. These control experiments established that our conditions were adequate to detect incorporation of ^{18}O upon hydrolysis of the globin adduct.

Globin from rats treated with [5-^{3}H]NNK was reacted with $NaCNBH_3$, dialyzed, and hydrolyzed with $Na^{18}OH$ in $H_2{}^{18}O$. Diol 7 was isolated by HPLC, silylated, and analyzed by GC–MS (see Figure 4). The molecular ion of the bis-TMS ether of 7 is m/z 311. The ratio of m/z 311 to m/z 313 was the same in the material isolated from

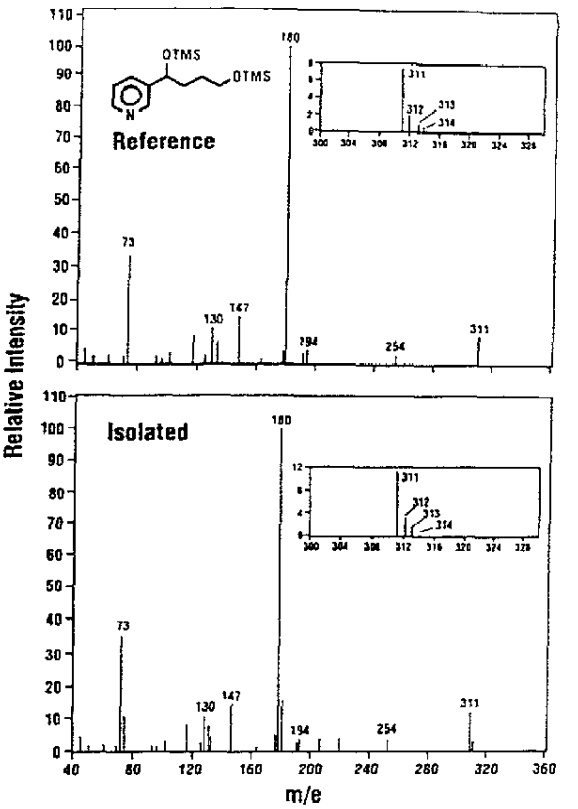

Figure 4. EI-MS of reference bis-TMS ether of diol 7 and material isolated from a 0.5 N $Na^{18}OH$ hydrolysate of $NaCNBH_3$-treated globin from a rat injected with [5-^{3}H]NNK. Insets: Details of molecular ion regions.

globin as in the standard. Thus, there was no detectable incorporation of ^{18}O and the adduct must be a carboxylic ester.

Further evidence was obtained by synthesis of the model aspartate ester 13. Hydrolysis was carried out under mild basic (0.15 N NaOH) or acidic (0.2 N HCl) conditions. Release of HPB was complete in base, whereas only 6% was released in acid. The release of HPB from a sample of globin from NNK-treated rats accounted for 19.6% of the radioactivity bound to globin when the hydrolysis was performed with 0.15 N NaOH while only 2.3% of the bound radioactivity was released as HPB in 0.2 N HCl (*5*). Therefore, the extent of hydrolysis was 12% as great in acid as observed in base.

Discussion

Previous studies have presented evidence for carboxylic ester formation in hemoglobin of laboratory animals treated with dimethyl- and diethylnitrosamine (*13*, *14*). These studies demonstrated the presence of methanol or ethanol in base or acid hydrolysates of hemoglobin. Esters would be likely products of the reaction of hemoglobin with methane- and ethanediazohydroxide, and the release of alcohols by hydrolysis of ester adducts would be expected. The release of HPB upon base or acid hydrolysis of hemoglobin from laboratory animals or humans exposed to NNK is consistent with these findings. However, the presence of the carbonyl group in the NNK molecule complicates assignment of the type of adduct. For example, we have shown that the cyclic cysteine adduct 14 is readily hydrolyzed to HPB, whereas the open-chain adduct

15 is stable to mild hydrolytic conditions (5). In addition, Schiff base formation is a possibility. Thus, the chemistry of NNK–hemoglobin adducts may be more complex than that of adducts formed from simple nitrosamines. Therefore, we carried out the ^{18}O-labeling experiments and model studies described above. The results provide unambiguous evidence that the HPB-releasing hemoglobin adduct is a carboxylic ester.

Labeling experiments with ^{18}O have also been used to establish the presence in hemoglobin of carboxylic ester adducts of benzo[*a*]pyrene-7,8-diol 9,10-epoxide (*15*). In this instance, a distinction could be made between the $B_{AL}1$ mechanism which occurs at pH 7–8.5 and results in ^{18}O incorporation into the benzo[*a*]pyrene tetraol hydrolysis product and the $B_{AC}2$ mechanism which occurs at pH >9 and does not result in any incorporation of label. In our case, it was not possible to examine the $B_{AL}1$ mechanism, even though it may be operative through assistance of the adduct carbonyl group, because this group would have undergone exchange with ^{18}O.

The carboxylic ester adduct could be formed by reaction with aspartate, glutamate, or the terminal carboxyl groups of globin. A recent study has used 1-amino-2,3-propanediol to locate one of the benzo[*a*]pyrene diol epoxide carboxylic ester adducts at aspartate(47) of the α chain of human hemoglobin (*16*). We are presently exploring this approach for identifying the acids involved in formation of the HPB-releasing adduct.

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